Brain tumour cell line authentication, an efficient alternative to capillary electrophoresis by using a microfluidics-based system

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Running title: Brain tumour cell line authentication

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Funding: This work was supported by Brain Tumour Research and Headcase.

Conflict of interest: All authors have declared that no conflicts of interest exist.

Abstract

Background. The current method for cell line authentication is short tandem repeat PCR (STR-PCR) -based genotyping involving co-amplification of a panel of STR loci by multiplex PCR and the downstream fragment length analysis (FLA), usually performed by capillary electrophoresis. FLA by capillary electrophoresis is time-consuming and can be expensive as the facilities are generally not accessible for many research laboratories. **Methods.** In the present study, a microfluidic electrophoresis system, the Agilent 2100 Bioanalyzer, was utilised to analyse the STR-PCR fragments from 10 human genomic loci of a number of human cell lines, including 6 gliomas, 1 astrocyte, 1 rhabdomyosarcoma, 1 primary lung cancer and 1 lung brain metastatic cancer; and this was compared to the standard method, i.e. capillary electrophoresis, using the Applied Biosystems 3130*xl* Genetic Analyzer. **Results.** The microfluidic electrophoresis method produced highly reproducible results with good sensitivity in sizing of multiple PCR fragments and each cell line demonstrated a unique DNA profile. Furthermore, DNA fingerprinting of samples from 5 different passage numbers of the same cell line showed excellent reproducibility when FLA was performed with the Bioanalyzer, indicating that no cross-contamination had occurred during the culture period. **Conclusion.** This novel application provides a straightforward and cost-effective alternative to STR-based cell line authentication. In addition, this application would be of great value for cell bank repositories to maintain and distribute precious cell lines.

Keywords: brain tumour cell authentication, STR profiling, DNA fingerprinting, microfluidics.

Introduction

The importance of cell line authentication has been well recognised by the research community and recently a list of 360 cross-contaminated and misidentified cell lines has been published, with the list being updated when necessary.[1] At present the widely proposed technology for human cell line authentication is short tandem repeat PCR (STR-PCR) -based DNA genotyping and the standard method for analysing STR fragments, i.e. fragment length analysis (FLA), is capillary electrophoresis.[2-5] There are a number of commercially available STR kits commonly used that provide convenient multiplex PCR amplification of the markers.[4] The downstream FLA is, however, normally done by capillary electrophoresis which requires state-of-the-art facilities and properly trained specialists to analyse the data obtained, resulting in outsourcing of the cell authentication service for the majority of research laboratories. We were interested in exploring the possibility of using other electrophoresis platforms for FLA, e.g. the Agilent 2100 Bioanalyzer that is a microfluidics-based Lab-on-Chip system for sizing and quantification of DNA, RNA, proteins and cells.[6-9] It also provides quality control of DNA, RNA and protein samples in a broad range of molecular assays.[10-12] Recently the Bioanalyzer has been employed to resolve STR fragments in forensic samples as well as to identify fish species based on their restriction fragment length polymorphism pattern.[13,14]

Our Cellular & Molecular Neuro-oncology Research Laboratories at the University of Portsmouth acts as the brain tumour cell culture repository for the South of England Brain Tumour Alliance (SEBTA), a network formed in 2011 by 7 regional centres in the South of England that are involved in diagnosis, treatment and research in the field of Neuro-oncology (http://sebta.org). With its clearly identified strategic aims SEBTA promotes and facilitates increased collaboration between those centres, including sharing valuable tissues and cell lines. Therefore, apart from our own group, we are responsible for the authentication of cell

lines that are distributed across the SEBTA centres by our brain tumour cell culture repository, and it is critical for us to establish an efficient workflow for the routine cell authentication tests. For that purpose, in the present study we investigated the potential of this microfluidic system in STR genotyping of human brain tumour cell lines, exploiting its capacity of DNA fragment separating and sizing. The microfluidics-based electrophoresis proved to be an effective and simple method for FLA in replacement of capillary electrophoresis. In addition, it created accurate and reproducible DNA profiling data for the cell lines studied, offering an efficient and reliable tool for our routine cell line genotyping test.

Materials and methods

Cell lines

All primary cell lines were derived from patient biopsy materials under Ethics permissions LREC 00-173 or KCH 11-094 or 11/SC/0048 in accordance with the National Research Ethics Service (NRES). These cell lines in addition to four established commercially available cell lines were used in the first part of the DNA profiling studies. The primary glioma cell lines included a grade II astrocytoma UP-016 / Passage (P)2, a grade III astrocytoma UP-032 (P5), three grade IV glioblastoma multiforme (GBM) cell lines, IN699 (P17), UP-019 (P9) and UP-029 (P5). One primary lung brain metastatic cell line, UP-024 (P4), was also included in this study. Commercially available cell lines analysed were: the GBM cell line SNB-19 (DSMZ – German Collection of Microorganisms and Cell Cultures; P63), the rhabdomyosarcoma cell line TE-671 (American Type Culture Collection (ATCC); P22), the human lung carcinoma cell line NCI-H1299 (American Type Culture Collection (ATCC); P25) and the normal human astrocyte cell line CC2565 (Lonza Biologics; P10).

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% foetal calf serum (FCS) (Sigma-Aldrich) with the exception of NCI-H1299 cells that were cultured in RPMI-1640 medium (Sigma-Aldrich).

To monitor potential changes (in particular cross-contamination) in cell lines over long-term culture, UP-029 and another biopsy-derived GBM cell line, SEBTA-003 (cultured in 10%FCS/DMEM), were genotyped in the second part of these studies. Cells were cultured for 5 continuous passages with 5-passage interval (i.e. P10, P15, P20, P25 and P30 of UP-029; P8, P13, P18, P23 and P28 of SEBTA-003).

STR-PCR

Genomic DNA was extracted from cultured cells using the QIAamp DNA Mini Kit (Qiagen). The STR-based multiplex PCR was carried out with the StemElite ID System (Promega) according to the manufacturer's protocol.[15] DNA amplification was set up in a final volume of 25µl reaction, including 1×Enzyme Mix, 1×Primer Pair Mix and 2ng of template DNA. PCR was performed on the MyCycler Thermal Cycler (Bio-rad) using the thermal cycling programme recommended for the Applied Biosystems GeneAmp PCR System 9600 Thermal Cycler, according to the StemElite ID System Manual (Promega).[15] The step-by-step thermal cycling protocol is as follows: step 1: 96°C for 2 minutes; step 2: 94°C for 30 seconds; step 3 (×10 cycles): ramp 60 seconds to 60°C and hold for 30 seconds, then ramp 50 seconds to 70°C and hold for 45 seconds; step 4: 90°C for 30 seconds; step 5 (×22 cycles): ramp 60 seconds to 60°C and hold for 30 seconds, then ramp 50 seconds to 70°C and hold for 45 seconds; step 6: 60°C for 30 minutes. The primers of the StemElite ID System Kit amplify 9 human STR loci, including D21S11, TH01, TPOX, vWA, CSF1PO, D16S539, D7S820, D13S317 and D5S818, as well as Amelogenin for gender identification. These markers are selected from the set of core STR loci that has been approved by the human identity testing

community.[4,16] STR-PCR was performed in 3 independent experiments for all cell lines in the first part of the studies (Exp.1, 2 and 3), whereas one STR-PCR experiment was carried out for 5 continuous passages of UP-029 and SEBTA-003, respectively, in the cross-contamination test study. A negative PCR reaction was included in each of the experiments where template DNA was replaced with water.

FLA

Amplified alleles in the STR-PCR products were separated on the Agilent 2100 Bioanalyzer (Agilent Technologies) with the Agilent DNA 1000 Kit (Agilent Technologies), according to the standard protocol with slight modifications.[17] In essence, the DNA chip was first primed with 9µl of the gel-dye mixture then 3µl of the PCR product was added to each of the 12 sample wells along with 3µl of the internal marker (Agilent Technologies). One microlitre of the Agilent DNA 1000 ladder (Agilent Technologies), used as a sizing standard, was loaded into the ladder well with 5µl of the internal marker. After electrophoresis, the amplified alleles from each sample were analysed using the manufacturer's software, i.e. 2100 Expert, provided with the instrument; the size of each fragment was determined based on the ladder and internal standards. In addition, the STR markers were identified based on their size ranges.[15] FLA was done with the Bioanalyzer for all samples from 3 independent STR-PCR experiments (Exp.1, 2 and 3). In comparison, samples from the first experiment (Exp.1) were sent to Eurofins MWG Operon (http://www.eurofinsgenomics.eu) and analysed using standard procedures of capillary electrophoresis on the Applied Biosystmes (ABI) 3130*xl* Genetic Analyzer, as described in the service information document (Eurofins MWG Operon).[18] The relevant negative sample was also included in each of the FLA analyses as a control.

Results

DNA profiling of cell lines by microfluidics-based electrophoresis and capillary electrophoresis

To assess the application of the microfluidics-based electrophoresis method in STR-based DNA profiling of human cell lines, amplified STR fragments of the above 10 cell lines from the first experiment (Exp.1) were separated and analysed by both microfluidic and capillary electrophoresis. The former was performed in our laboratory using the Agilent DNA 1000 Kit and the data were analysed by the Agilent 2100 Expert software; the latter by Eurofins MWG's FLA service using the ABI 3130*xl* Genetic Analyzer as recommended for the StemElite ID System.[15] Amplified STR fragments from all 10 cell lines were successfully separated by the Bioanalyzer as demonstrated by the representative electropherograms (Fig. 1**A** & **B**). Although DNA fragment sizing was on average around 20bp larger than that obtained from capillary electrophoresis, likely due to the dye-labelled PCR primers which had incorporated into the fragments and consequently reduced the migration speed of those fragments, both sets of data were highly comparable in terms of the DNA profiles of the cell lines studied (Table 1**A**). There were differences in several of the STR markers when comparing these two methods. When analysing the STR marker D21S11, 4 of the 10 cell lines using the microfluidic method failed to detect the heterozygous alleles compared to capillary electrophoresis (shaded cells in Table 1**A**). The STR markers, D5S818, vWA, TPOX and CSF1PO, showed homozygous alleles in 10% (1/10) of the cell lines where capillary electrophoresis revealed heterozygosity of the associated marker in those cell lines (shaded cells in Table 1**A**). On the contrary, the microfluidic system was highly consistent in terms of analysing homozygous SRT markers and the homozygosity matched 100% between those two methods (Table 1A). In one cell line (UP-029), there was a discrepancy in the sizes of two alleles of D7S820 between the methods, with 1bp difference in the microfluidic

method whereas there was 16bp by capillary electrophoresis (underlined cells in Table 1**A**). In total, 92% (92/100) of the analysed markers showed excellent similarity between the Bioanalyzer and capillary electrophoresis in terms of fragment sizing. Importantly, both microfluidic and capillary electrophoresis revealed a unique DNA profile of each cell line, presented by fragment sizing for the former (Table 1**A**) and by the internationally recognised standard for the latter (Table 1**B**).

The DNA profiles of NCI-H1299 and TE-671 in the present study matched the ones published by ATCC[19] and in the DSMZ database respectively (Table 1**C**). While previous STR analysis at ATCC revealed that the human GBM cell line SNB-19 had an identical profile to U-373 MG, another GBM cell line, which led to SNB-19 being discontinued by ATCC.[20] As mentioned above, our laboratory obtained the SNB-19 from the DSMZ – German Collection of Microorganisms and Cell Cultures and this cell line was included in the DNA profiling study to determine if our SNB-19 cell line had been cross-contaminated. Our data confirmed that the SNB-19 cell line in our cell bank was not cross-contaminated with U-373 MG as reported by ATCC, based on the comparison between STR profiles of our SNB-19 cell line and the reported U-373 MG (Table 1**C**).[21] According to the DSMZ data sheet, SNB-19 is a "subclone" of the human GBM cell line U-251 MG.[22] This conclusion is supported by previous studies indicating that those two cell lines carry 96% genotype similarity and identical genomic variants, i.e. TP53, CDKN2A and PTEN mutations,[16,23] although SNB-19 and U-251 MG have diverged at the karyotypical level.[24] Interestingly, when STR matching analysis was performed using DSMZ's online tool for the SNB-19 cell line included in the present study, the result shows that our SNB-19 has the identical STR profile to those for U-251 MG and SNB-19 in the DSMZ database (Table 1**C**). Hence the SNB-19 cell line cultured in our laboratories has 100% genotype similarity to both cell lines (i.e. U-251 MG and SNB-19) held at DSMZ. Our data also provide further evidence for the

previous observation that SNB-19 is a derivative of U-251 MG. In addition, the STR profiles of three commercial cell lines, NCI-H1299, SNB-19 and TE-671, obtained by capillary electrophoresis in our study were identical to those in published databases (Table 1C), indicating good reproducibility of the conventional method.

Reproducibility of FLA using a microfluidics-based electrophoresis system

In order to evaluate the reproducibility of this alternative electrophoresis platform in the application of STR fragment length analysis, three independent experiments were carried out with multiplex PCR amplification and fragment separation/sizing for the 10 cell lines in the DNA profiling study, using the same DNA sample of each cell line. Except D5S818 in UP-016 and D21S11 in IN699 (shaded cells in Table 2), 98% (294/300) of the STR markers analysed amongst three experiments showed ±1-3bp difference in their sizes, indicating an excellent reproducibility of the Bioanalyzer in FLA analysis (Table 2). Interestingly, fragment sizes revealed in Exp. 3 were slightly larger in general for the majority of samples compared with those in Exp.s 1 & 2 (Table 2), caused by slightly variable electrophoresis migration rates in those assays; however, due to the same trend of sizing differences demonstrated by all STR markers (instead of only one or two markers), the whole STR profile was maintained despite slight shifting of the profile between different experiments. Hence all cell lines showed highly identical DNA profiles from three independent experiments, as represented by IN699 and SNB-19 (Fig. 2**A** & **B**).

Consistent DNA profiling of cell line over multiple subcultures

Cross-contamination in cell culture invalidates research results and compromises the comparison between laboratories. To further validate the application of the microfluidic system in cell line DNA profiling and to detect possible cross-contamination during long-

term cell culture, the same STR-based cell identity test was carried out with five genomic DNA samples collected from each of the two brain tumour cell lines, i.e. UP-029 and SEBTA-003, with 5-passage interval respectively. As the data indicated, each cell line showed consistent STR profiles throughout five different passage numbers with ±1-3bp difference in sizing amongst 10 markers (Table 3**A** & **B**), suggesting that no cross-contamination occurred during the *in vitro* culture period and each cell line had maintained its unique DNA fingerprint. The overlaid DNA profiles of 5 different passages further confirmed the identical STR fingerprints of the cells (Fig. 3**A** & **B**). In accordance with the initial DNA profiling data, the sizing of all 10 markers in the cross-contamination test showed ±1-3bp difference amongst different passages (Table 3**A** & **B**). Therefore, our data indicate that DNA profiling by microfluidic electrophoresis could be an effective approach to monitoring cross-contamination over time and subcultures. Notably, UP-029 was also included in the first part of this DNA fingerprinting study where a different Agilent DNA 1000 kit was used, and the profile of UP-029 cells maintained excellent similarity throughout different experiments/kits. These data provide further evidence for the reliability of this method and the Agilent assay kits in the application of cell line authentication.

In addition, the StemElite ID System used in this study also incorporates a sensitive mouse marker that can be detected after multiplex PCR should the sample contain genomic DNA from mouse;[15] and the data suggest that no mouse contamination was detected in all cell lines analysed in the present study (data not show).

Discussion

The present study demonstrated a new application of the microfluidics-based electrophoresis method in STR-based DNA profiling of human cell lines, as shown here with brain tumour and brain tissue related cell lines. In this study, the Agilent 2100 Bioanalyzer was used to

analyse the STR fragments and compared with the standard capillary electrophoresis method. Since its launch in 1999, the Bioanalyzer has been utilised for quality check and quantification of DNA and RNA samples before downstream applications such as sequencing and microarray analyses.[11, 25-28] Due to its capacity of separating multiple DNA fragments within the same sample, it has been utilised in STR-based DNA genotyping of forensic samples.[13] The Agilent 2100 Bioanalyzer offers a broad range of pre-validated analysis kits, including the DNA 1000 chip that was used in this study. This DNA chip provides 5-25bp sizing resolution for fragments of 100-500bp.[17] The size range of the STR markers in the StemElite ID System is between 90 to 400bp;[15] therefore it had been speculated that those amplified alleles would be separated on the DNA 1000 chip. Notably, the StemElite ID System is designed specifically for FLA by the Applied Biosystems capillary electrophoresis instruments, e.g. the ABI 3130 and 3130*xl* Genetic Analyzer.[15] Here our data demonstrate that this cell identification kit is valid for use with a microfluidic chip system, the Agilent 2100 Bioanalyzer. The STR profiles obtained from both FLA methods were highly comparable in this study, despite some discrepancies in the heterozygosity of several markers (Table 1A; shaded cells). This could have been caused by unbalanced PCR amplification of those markers, i.e. one allele was preferentially amplified than the other, and therefore the Bioanalyzer failed to detect the other allele due to low sample concentration whereas the capillary electrophoresis system was able to capture the fluorescent signal and detect the labelled fragment. The multiplex PCR in this study was performed according to the manufacturer's protocol for the StemElite ID System[15] but in the future analyses, the amplification parameters could be modified in order to enhance balanced amplification of the markers and subsequently improve the resolution of this novel method in detecting heterozygous alleles. Notably, only 8% (8/100) of the total markers analysed showed a

discrepancy in the heterozygosity between the new and conventional methods and this had not affected the unique profile of each cell line studied (Table 1A).

In summary, the microfluidic system produced highly accurate and reproducible DNA profiling data from 10 different cell lines demonstrated in the present study, suggesting a new approach to cell line authentication using this platform. Furthermore, the comparison analysis featured by the Bioanalyzer 2100 Expert software allows a quick fingerprint check for different DNA samples from the same cell line by overlaying DNA profiles from either independent or the same DNA chip assays, as demonstrated in Fig.s 2 and 3. This analysis can also be performed for any two cell lines in order to compare and confirm their different profiles, as illustrated in Fig. 1**C** for TE-671 and UP-019 showing distinct profile of each cell line. This could provide a reliable and cost-efficient downstream method for STR-based cell line identity check in a cell culture laboratory.

It has been fully recognised that routine identity check should be conducted for human cell line cultures and recently the importance of cell line verification prior to scientific publication has been reiterated by Anja Torsvik *et al*, as the authors discovered that the previously reported spontaneous transformation of human mesenchymal stem cells was in fact caused by cell line cross-contamination.[29] Their report also sheds new light on the urgent need for cell validation test of primary cell cultures for therapeutic purposes.[29] In the present study, the microfluidics-based method was employed to monitor possible cross-contamination over long-term culture period and an excellent accuracy of this novel method was illustrated by the identical DNA profiles obtained from different passages of the same cell line (Table 3 & Fig. 3), indicating the potential of this method as a more effective alternative for cross-contamination check. However, further studies need to be carried out to evaluate and compare the sensitivity and specificity of both FLA methods in terms of

detecting cross-contamination, using carefully designed mixed cell cultures to represent the most common contamination scenarios.

Recently new standards for cell line authentication using STR fingerprinting has been proposed.[1-5,30] It is critical that an internationally standardized STR panel is applied in the cell line identity test. The StemElite ID System employed in this study comprises 9 STR markers which are in accordance with the markers utilised by ATCC and DSMZ, allowing comparison of the fingerprints of our NCI-H1299 and SNB-19 cells with those in the above large databases. Notably, a recent study by Pierre Bady and colleagues suggests that the commonly used similarity score of 0.8 is not sufficiently stringent to differentiate the origin of a cell line based on its DNA fingerprint consisting of only 9 STR loci[26] and it was proposed that the number of STR markers measured should be expanded or to include additional cell line characteristics, e.g. specific mutations.[31] The focus of this current study was to evaluate an alternative method for the standard fragment length analysis by capillary electrophoresis, which is time-consuming and costly for most laboratories. Both methods, however, are adaptable for expansion of STR markers. The DNA fingerprints of two commercially available cell lines recruited in the present study, i.e. NCI-H1299 and SNB-19, demonstrated 100% similarity in comparison with the 9-marker fingerprint database of ATCC and DSMZ, confirming the origins of those cell lines held in our laboratories. The DNA profile of CC2565, another commercially available cell line analysed in this study, was compared with the DSMZ database with no matching profile revealed (data not shown), indicating that the STR profile of CC2565 is not included in this database. Each of the 7 "in-house" cell lines presented in this study showed a unique DNA profile, suggesting that the 9-marker approach was sufficient to identify and differentiate those biopsy-derived cultures.

It is likely that there will be a need in the future to expand the panel of STR markers with increasing number of 'in house' cell lines to be tested and this method will

accommodate this need. We have shown that this microfluidics-based electrophoresis platform could provide a more straightforward and cost-effective method for STR-based cell line authentication test in our hands, compared with the standard capillary electrophoresis for DNA fragment length analysis. It is our hope that this novel application may promote an increase in the routine cell line validation test not only by cell bank repositories and distributors, but also by the research community. In addition this method could be useful in a broader range of applications, e.g. testing human cancer cells directly passaged and maintained *in vivo* using mouse xenograph models and validating cells used in clinical cell therapies. It is also our intention to apply this method in our future studies to address several key research questions, in particular with regard to monitoring genetic instability caused by *in vitro* culture, assessing cross-contamination susceptibility levels in different cell lines and developing personalised medicine.

Funding: This work was supported by the brain tumour charities Brain Tumour Research and Headcase.

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Figure legends

Fig. 1. Representative single (**A** & **B**) and comparison (**C**) electropherograms of DNA profiling by a microfluidic system, the Agilent 2100 Bioanalyzer. STR profiles of the cell lines TE-671 (**A**) and UP-019 (**B**) are illustrated as DNA electrophoresis traces here. Fragments of the PCR-amplified STR markers are separated based on their sizes and the size of each peak is labelled above it in panels **A** and **B**. The overlaid traces of TE-671 and UP-019 in panel **C**, obtained from the "Comparison" analysis (Agilent 2100 Expert software), show the unique STR profiles of these two cell lines.

Fig. 2. Overlaid DNA profiles of IN699 (**A**) and SNB-19 (**B**) from three independent analyses by the microfluidic system. All cell lines analysed in the three independent experiments showed reproducible STR profiles, in particular between Exp.1 and Exp.2, as represented by IN699 and SNB-19.

Fig. 3. Overlaid DNA profiles of UP-029 (**A**) and SEBTA-015 (**B**) from 5 continuous passage numbers, showing the identical profile throughout different passages.